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A Library Approach to the Generation of Bisubstrate Analogue Sulfotransferase Inhibitors

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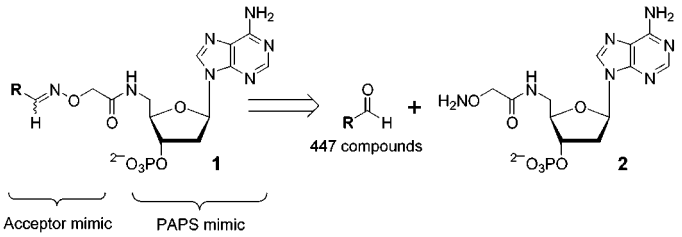
ABSTRACT



A library of potential bisubstrate analogue inhibitors (1) targeting sulfotransferase enzymes was generated by the chemoselective ligation of the PAPS mimic 2 with a panel of 447 aldehydes. Preliminary screening has identified compounds that inhibit estrogen sulfotransferase (EST), an enzyme relevant to breast cancer.

Sulfated biomolecules mediate a wide array of normal and pathological cellular events. For example, sulfated carbohydrates arbitrate leukocyte adhesion to inflamed endothelium,[1] sulfated proteins modulate HIV-1 infectivity,[2] and sulfation also regulates the activity of steroid hormones in vivo.[3] The role of sulfated biomolecules in numerous diseases has prompted the search for inhibitors targeting the enzymes that install sulfate esters, the sulfotransferases.

Sulfotransferases catalyze the transfer of a sulfuryl group from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to a hydroxyl (or amino) group on a carbohydrate,[4] protein,[5] or small molecule[6] acceptor (Figure 1). Over 25 human carbohydrate and protein sulfotransferases have been identified, including many homologous isozymes with similar substrate specificities and overlapping tissue distributions. The strong connection between sulfotransferases and several disease states has prompted interest in the discovery of potent and selective sulfotransferase inhibitors both as tools to elucidate the role of these enzymes in vivo and as potential therapeutics.[7]

We have been exploring the inhibitory potential of PAPS-competitive compounds derived from substances traditionally used to target the ATP-binding pocket of protein kinases.[8] Although we have found drug-like PAPS-competitive com-

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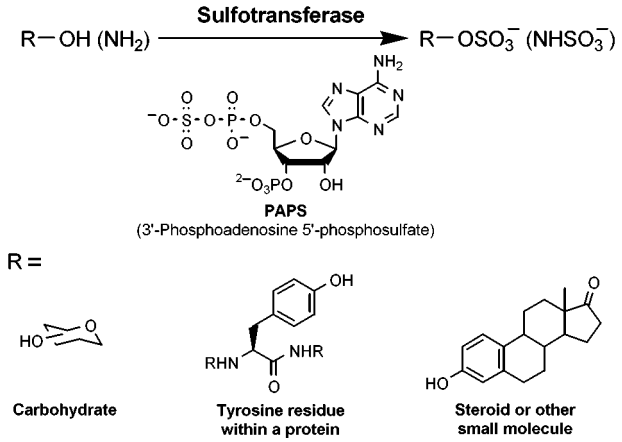


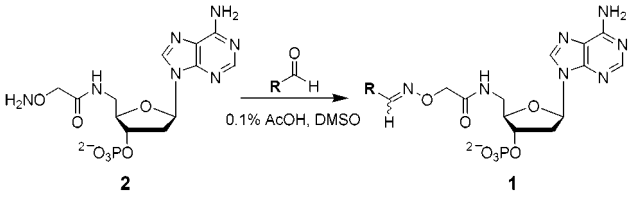
Figure 1. Sulfotransferases catalyze sulfuryl-group transfer from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to biomolecules.

pounds with moderate to high potency in vitro,[8] we anticipate difficulty in achieving selective inhibition of related sulfotransferase enzymes in vivo since the family of sulfotransferases shares a similar PAPS-binding pocket. To surmount this problem, we focus here on the preparation of bisubstrate analogues.

Bisubstrate analogues can provide specificity via key interactions within both binding pockets of the enzyme.[9] This is a clear advantage in the case of sulfotransferases that share high sequence similarity in the PAPS-binding region but possess disparity in other regions including the acceptor-binding site (Figure 2a).[10] In addition to specificity, potency is achieved from the entropic advantage of linking structures that mimic each substrate (Figure 2b). Many of the traditional design strategies for bisubstrate analogues that target group transfer enzymes, such as kinases[11] and glycosyltransferases,[12] result in potent inhibitors in vitro. However, the compounds are usually too hydrophilic or charged to cross cell membranes and are therefore not useful for cellular studies.

Our strategy focused on the design of a library possessing two elements: (1) a PAPS mimic that directs the compounds to sulfotransferases and (2) a variable component that is hydrophobic and drug-like to fill the acceptor-binding pocket (Figure 2c). The target library, structure **1** (Scheme 1),

Scheme 1



contains an oxime linkage between the elements, allowing the parallel synthesis of hundreds of potential bisubstrate

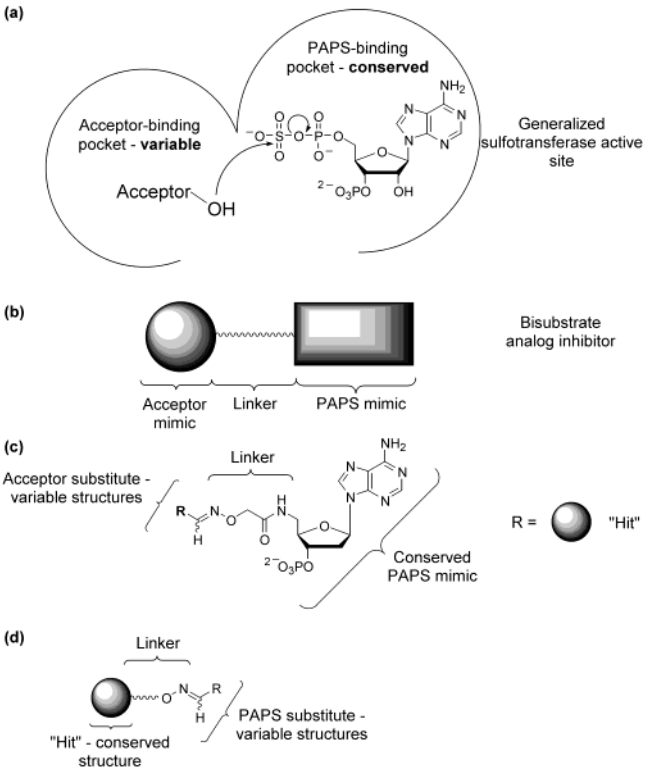


Figure 2. (a) Schematic of a sulfotransferase active site containing both the conserved PAPS-binding pocket and an acceptor-binding pocket with variable amino acid residues. (b) Generalized structure of a sulfotransferase bisubstrate analogue inhibitor. (c) The first phase of library design utilizes an aminooxy-functionalized PAPS mimic to identify pharmacophores that target the acceptor-binding pocket. (d) An aldehyde-derived "hit" from the first phase will be used as a scaffold to identify PAPS substitutes.

inhibitors via the chemoselective coupling of aminooxy nucleoside **2** with commercially available aldehydes (Scheme 1). The oxime-forming reaction tolerates numerous functional groups, an attribute that has been exploited for the chemoselective ligation of pharmacophores in a library synthesis,[13] attachment of sugars to peptides,[14] and the modification of cells.[15] Once a hit is identified, the library strategy can be reversed by using the aldehyde-derived acceptor-mimic as a scaffold; derivatizing this compound with an aminooxy linker would allow us to probe for more bioavailable inhibitors targeting the PAPS-binding site (Figure 2d). Ultimately, the two-step strategy may yield compounds with

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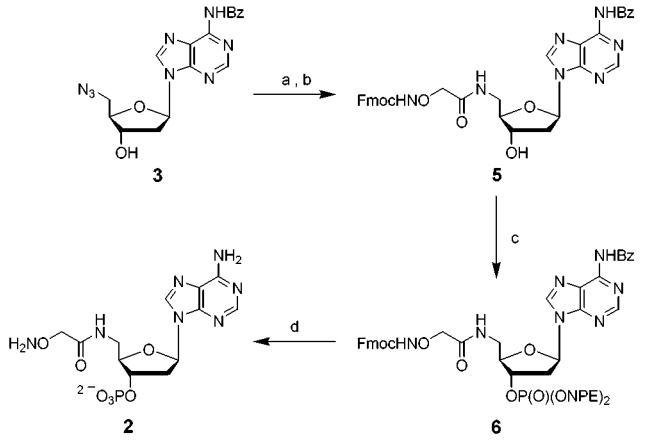
linked pharmacophores that simultaneously bind in both substrate-binding pockets.

As the first step toward this goal, we have synthesized **2** and used it to prepare library **1** consisting of 447 unique compounds. We chose the 2′-deoxyadenosine scaffold after evaluating the X-ray crystallographic data for both EST[16] and the sulfotransferase domain of heparin *N*-deacetylase/*N*-sulfotransferase[17] and finding no key interactions between the enzyme active site residues and the 2′-hydroxyl group of PAPS. In addition, the 2′-deoxy analogues are more hydrophobic and more synthetically tractable, requiring fewer protecting group manipulations to install the 5′-aminooxy linker.

The new PAPS analogue **2** was prepared from the azidonucleoside **3**[18] (Scheme 2) by a Pd-mediated reduction

Scheme 2[a]



[a] Reagents and conditions: (a) 10% Pd/C, AcOH, MeOH (62%); (b) $FmocNHOCH_2COOH$ (**4**), EEDQ, DMF (63%); (c) (i) $(i\text{-}Pr)_2NP(ONPE)_2$, tetrazole, CH_2Cl_2; (ii) *m*CPBA (71%); (d) (i) $n PrNH_2$, CH_3CN, 1 h; (ii) BSA, DBU (57%).

of the azido group to the amine followed by coupling with 2-[(*N*-fluorenemethyloxycarbonyl)aminooxy]acetic acid (**4**)[19] to provide **5**. Standard phosphoramidite chemistry was then employed using di(*p*-nitrophenethyl (NPE)) diisopropylphosphoramidite[20] followed by oxidation with *m*CPBA to provide the phosphorylated nucleoside **6**. Complete deprotection was achieved via the stepwise addition of excess *n*-propylamine to a solution of **6** (100 mM, CH_3CN) followed by treatment with bis(trimethylsilyl)acetamide (BSA) and 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU). Purification of **1** via reversed-phase HPLC eluting with a gradient of 0 to 20% CH_3CN in 25 mM NH_4OAc provided the ammonium salt of **2** in 57% yield.

Library synthesis was performed in a 96-well plate format using 25 mM **2** (DMSO containing 0.2% AcOH) and 20 mM aldehyde (DMSO, 447 aldehydes total). The reactions were incubated at room temperature in the dark for 48 h before they were frozen for later use. Approximately 10% of the library was analyzed by reversed-phase HPLC, and the major peak was isolated and characterized by electrospray ionization mass spectrometry. The reaction yields were typically high (>90%) as determined by a reduction in peak intensity for **2**. However, more than one product peak was observed in approximately 10% of the 40 reactions analyzed, precluding the determination of an accurate yield via this method.

The library was then screened for inhibitors of the cytosolic enzyme, estrogen sulfotransferase (EST), using the previously developed immobilized enzyme mass spectrometry (IEMS) assay.[21] Unusually high levels of sulfated estrogen are found in breast cancer cells; thus EST inhibitors are of potential therapeutic value.[22] Briefly, EST was immobilized on agarose beads via reductive amination and a mixture of the library components was analyzed by mass spectrometry both before and after exposure to the immobilized enzyme. A reduction in peak intensity for a library component indicated binding to the enzyme. Figure 3 shows the mass spectrometry data,

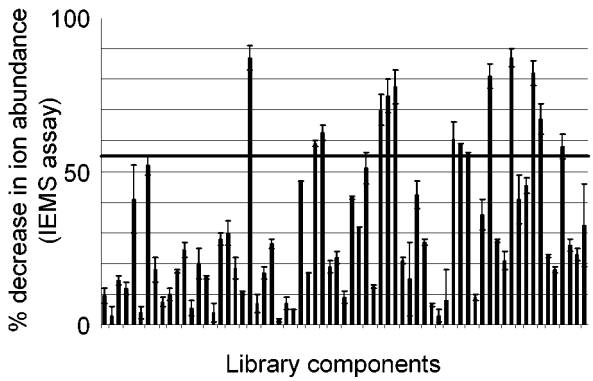


Figure 3. Results from the IEMS screen (performed in duplicate) of the library (subset of 67 members) against EST. Compounds displaying greater than a 55% decrease in ion abundance after enzyme incubation were considered "hits." Error bars indicate the result of each replicate.

expressed as percent (%) decrease in peak intensity for 67 compounds. We have previously demonstrated a strong correlation between a reduction in peak intensity and inhibitory activity,[8b] and we were interested in establishing this correlation for library **1**. We therefore screened the 67 compounds for EST inhibitory activity using a previously developed radiolabel-transfer TLC assay.[8b] We found that all compounds displaying greater than 50% inhibition at 200 μM also shared greater than 55% ion abundance decrease in the IEMS assay (Figure 4). Although several of the "hit"

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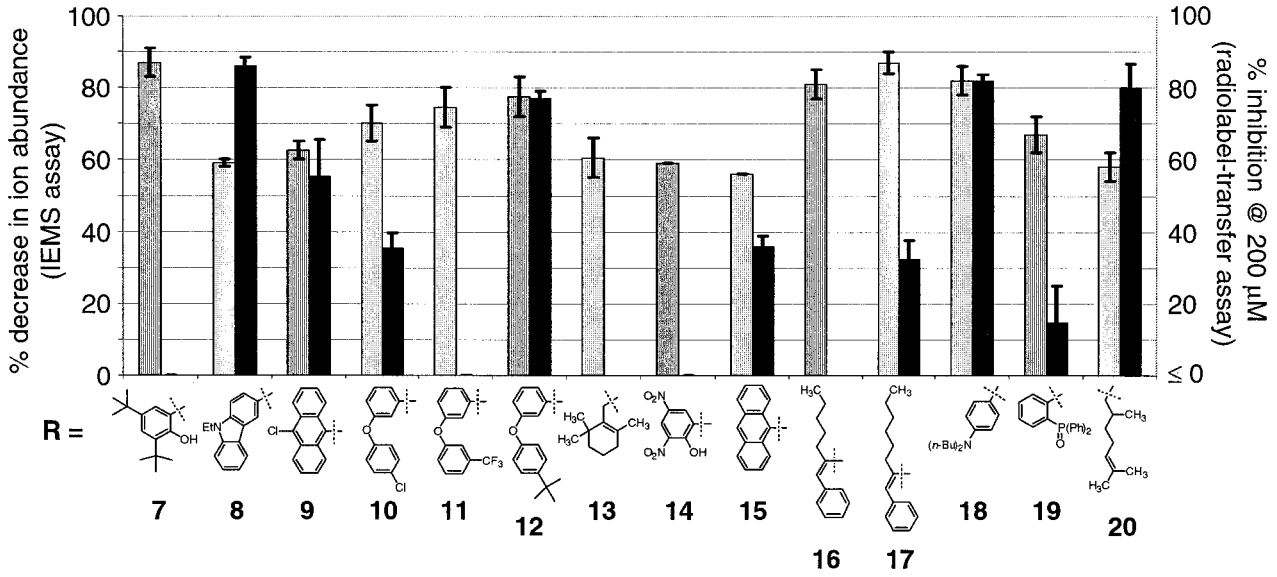

Figure 4. A comparison of the IEMS binding data (shaded bars) and radiolabel-transfer inhibition data (dark bars) reveals several EST inhibitors. Compounds **8**, **12**, **18**, and **20** were confirmed as hits. False positives ("hits" from the IEMS assay, but not from the radiolabel-transfer assay) are expected since compounds may bind to the enzyme without inhibiting activity. Examples are compounds **7**, **11**, **13**, **14**, and **16**. Each assay was performed in duplicate; the error bars represent the result of each replicate. The R groups correspond to substituents in compound **1**.

compounds in the IEMS assay lacked inhibitory activity (compounds **7**, **11**, **13**, **14**, **16**), it is noteworthy that the IEMS assay successfully identified all of the inhibitors revealed by the radiolabel-transfer TLC assay. Specifically, compounds **8**, **18**, and **20** demonstrated significant inhibitory activity (>80% inhibition) against EST. Surprisingly, these compounds vary widely in aldehyde structure. This implies that the aldehyde structures bind to disparate regions of the active site or that the structures contain similar binding motifs that would have been difficult to rationally predict. Our strategy therefore reveals a "fingerprint" of pharmacophores that target the acceptor-binding pocket of EST. As we expand our efforts and screen **1** against a panel of sulfotransferases, we expect to generate "pharmacophore fingerprints" for each enzyme, allowing us to identify unique structures that bind with specificity to each acceptor-binding pocket.

We are currently screening library **1** against a panel of sulfotransferases to identify inhibitors for each enzyme and determine the selectivity of the "hits". With the most potent inhibitors in hand, the strategy will be reversed, using the aldehyde-derived moiety as an anchor to probe the PAPS-binding pocket. This approach will hopefully yield cell permeable, specific, and potent sulfotransferase inhibitors for use in future in vivo studies.

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Supporting Information Available: Experimental procedures and spectral data for all new compounds and the structures of all aldehydes used to generate library **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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